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<p>(54) Title: PORPHYRIN COMPOUNDS, THEIR CONJUGATES AND ASSAY METHODS BASED ON THE USE OF SAID CONJUGATES</p> <p>(57) Abstract</p> <p>The present invention describes the preparation of phosphorescent metalloporphyrin labelling reagents and the use of said labelling reagents for preparation of phosphorescent conjugates with biomolecules. The labelling reagents obtainable according to this invention are water soluble monofunctional derivatives of Pt- and Pd-coproporphyrins, where the term 'monofunctional' refers to the number of reactive groups in the porphyrin moiety.</p>		

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## PORPHYRIN COMPOUNDS, THEIR CONJUGATES AND ASSAY METHODS BASED ON THE USE OF SAID CONJUGATES

### FIELD OF THE INVENTION

- 5 This invention relates to the chemistry and applications of the metalloporphyrins. In particular it is related to novel photoluminescent metalloporphyrin compounds, their preparation and to the use of the said compounds as labelling reagents for biologically active molecules. This invention can be applied for example, in biomedical research and in-vitro diagnostics, in food industry, in chemical and  
10 pharmaceutical industry, in biotechnology as well as in environmental monitoring.

### BACKGROUND OF THE INVENTION

- Many (bio)analytical systems utilise certain chemical compounds and species as sensitive and specific probes for the determination of specific analytes and parameters. In particular, photoluminescent dyes are frequently used for labelling of  
15 biomolecules in different analytical applications. Various photoluminescent dyes with different physical, chemical and spectral properties have been described, that suit a wide range of applications, such as staining of proteins, nucleic acids, cells, for use in microscopy, immunoassays and DNA hybridisation assays. The most common families of the dyes include fluoresceins, rhodamines, coumarines,  
20 cyanines and BODIPY dyes (=boron dipyrromethine dyes). Families of the dyes which enable high detection sensitivity include photoluminescent lanthanide chelates and phosphorescent metalloporphyrins. A large number of photoluminescent fluorescent dyes and their biomolecule conjugates, such as protein conjugates and nucleic acid conjugates, have been developed in recent  
25 years. Many photoluminescent dyes as well as the corresponding labelling reagents and biomolecule conjugates are available commercially (see for example R.P.Haugland, Handbook of fluorescent probes and research chemicals, 6<sup>th</sup> Edn.,

Molecular Probes Inc., 1996) and widely used for diagnostics and research purposes. Photoluminescent conjugates of biomolecules are usually prepared by contacting a native biomolecule or a derivative of the biomolecule with a reactive derivative of a photoluminescent dye, also called labelling reagent. Examples of reactive groups in common labelling reagents include reactive esters (for example succinimidyl ester, sulphosuccinimidyl esters), isothiocyanato, chlorosulphonato, dichlorotriazinyl and maleimidyl groups. They enable simple and selective labelling of biomolecules via certain functional groups, most commonly via primary amino, thiol or hydroxy groups. Labelling can be performed under mild conditions and without using additional reagents. The availability of such standard labelling reagents and the corresponding photoluminescent bioconjugates is a requirement for development of analytical and diagnostic methods and applications. In many cases it is desirable to covalently attach the dye molecule via a spacer arm (usually 2-20 atoms long), in order to minimise the photochemical and biochemical interactions between the label and the biomolecule.

For those applications where high detection sensitivity is required, such as immunoassays and nucleic acid hybridisation assays, probes with long Stokes' shift and well resolvable spectral characteristics are needed. Significant improvement of sensitivity, especially when working with complex biological samples, can be achieved by using long decay time photoluminescent dyes in combination with time-resolved photoluminescence detection. The time-resolved detection concept allows significant reduction of background interference from scattering and intrinsic fluorescence and consequently provides high signal-to-noise ratio (E. Soini et al. US Pat. No 4374120, 1983).

Long decay time photoluminescent lanthanide chelates, in particular the complexes of Eu, Tb, Sm, Dy have been suggested for use in sensitive time-resolved fluoroimmunoassays (for example US 4565790, US 5346996, US 5571897). They are presently used in a commercial time-resolved fluoroimmunoassay system DELFIA® (trademark of EG&G-Wallac, Turku, Finland). p-isothiocyanatobenzyl-

diethylenetriamine- $N^1, N^2, N^3, N^4$ -tetraacetic acid chelated with  $Eu^{3+}$  is now commercially available for labelling of biomolecules, such as proteins, antibodies and nucleic acids.

- Fluorescent complexes of ruthenium(II), osmium (II), and some other metals were suggested recently for use as long decay time fluorescent labels, particularly for oxygen sensors and for sensitive fluorescence immunoassays (J. Lakowicz et al., US 5660991). Succinimidyl derivatives of Ruthenium bis(2,2'-bipyridine) (2,2'-bipyridine-4,4'-dicarboxylic acid) have been described as labelling reagents for electrochemiluminescence immunoassays (US Pat. 5310687).
- 10 A number of photoluminescent porphyrin dyes have been suggested for labelling of biomolecules, particularly for immunoassays (Schmidt D. et al. US 4614723; Hendrix J., US 4707454; Hendrix J., US 5464,741; Savitskii A.P. et al., Doklady Akademii Nauk SSSR, 1987, vol. 293, p.744, Braman, J. WO 96/11937). These dyes exhibit intense absorption bands around 400 nm and moderate absorption
- 15 bands between 500 and 600 nm. Free base porphyrins usually emit fluorescence in red range of visible spectrum, whereas their metal complexes, mainly platinum(II) and palladium(II) porphyrins, exhibit bright phosphorescence in the same wavelength range even at room temperature (D.Dolphin, The Porphyrins, 1978, New York, Academic Press, vol. 3). Such properties make the porphyrin dyes
- 20 promising in fluorescent and phosphorescent probing of biomolecules and particularly in time-resolved phosphorescence bioaffinity assays. A number of porphyrins as well as certain related structures, mainly biogenic polycarboxy porphyrins and water-soluble derivatives of tetraphenylporphyrin, have been suggested for such applications.
- 25 Water-soluble coproporphyrin-I was suggested by Savitsky et al. (Doklady Akademii Nauk SSSR, 1987, vol. 293, p.744) as a label for fluoroimmunoassays. A palladium(II) complex of coproporphyrin-I has been used for phosphorescence labelling of antibodies (Doklady Akademii Nauk SSSR, 1989, vol. 304, p.1005). In

- similar way some other polycarboxy porphyrins, including coproporphyrin-III, zinc(II)-coproporphyrin-I, dimethoxydeuteroporphyrin IX, hematoporphyrin IX, platinum(II)-coproporphyrin I and III, have been used for labelling various proteins. With these dyes covalent binding to proteins was usually achieved by carbodiimide method with various modifications. In a two-step procedure the carboxy groups of the porphyrin were first activated by carbodiimide in aqueous solution or in organic media and then allowed to react with protein amino groups in slightly alkaline aqueous solution. A drawback of the methods where carbodiimides are used for activation of porphyrins having more than one carboxy group, is that a mixture of different products is formed as a result of the carbodiimide activation. The mixture comprises porphyrins having from 0 to n pieces of activated carboxy groups where n is the number of carboxy groups in the porphyrin before the activation. Multi-point activation of porphyrins often causes significant crosslinking and inactivation of biomolecules such as proteins when they react with such reagents.
- 15 An additional drawback of the carbodiimide chemistry is that it does not allow the active product to be isolated in pure form, but the product remains in form of undefined mixture. Furthermore, the active carbodiimide adduct is instable. It is sensitive to hydrolysis as well as to many other nucleophiles. Typically, the carbodiimide adducts react intramolecularly producing non-reactive N-acylurea.
- 20 The chemical instability of the carbodiimide-activated porphyrin adducts makes their use difficult and unreliable. Also porphyrin-antibody conjugates prepared by carbodiimide mediated coupling has been found to exhibit poor storage stability (Martsev et al., J.Immunol. Methods, 1995, vol. 186, p.293). The use of tetra-succinimidyl esters of coproporphyrins as labelling reagent did not provide
- 25 significant improvement in performance or stability of the conjugates (Martsev et al., J.Immunol. Methods, 1995, vol. 186, p.293).

In US 4,614,723 Schmidt et. al. introduced the use of porphyrins comprising several carboxy groups as label for immunoassays. The method of activation of porphyrins according to Schmidt's patent follows same carbodiimide chemistry as

described above. G. Sagner and R. Haas (EP 0811626 A1) describe a preparation method for labelling reagents from water-soluble platinum(II) porphyrins: platinum coproporphyrin-I (PtCP) and platinum tetra-(p-carboxyphenyl) porphine (PtTCPP). However, in the patent publication as well as in two other publication by R Haas (J. Histochem. Cytochem., vol. 45, p.1279 (1997), J. Histochem. Cytochem, vol. 47,183-196 (1999)) the detailed chemical structures and respective systematic names of the label remain ambiguous. For example, the systematic name of a compound which they trivially call as 'platinum coproporphyrin', has been written as 'Pt-3,8,13,18-tetramethyl-21H,23H porphine-2,7,12,17-tetrapropionic acid'. It is evident to a skilled person in the art, that upon insertion of a metal cation to the porphyrin cavity, a substitution occurs where the two hydrogen atoms bound to the positions 21 and 23 in the free base porphyrins are replaced. Thus, in case the authors mean phosphorescent metallo porphyrin chelate, the systematic name for the product should be 'Platinum (II) complex of 2,7,12,17-tetramethyl-21H,23H-porphine-3,8,13,18- tetrapropionic acid'. In EP 0811626A1, Sagner et. al., the labelling reagent was prepared by applying carbodiimide chemistry as applied in several other publications referred to above. The specific teaching of this Sagner's patent publication is that the activation reaction of the hydrophilic carboxy porphyrin has been carried out in dry organic solvent where hydrolysis of the activated derivatives should not take place. Preparation of the active porphyrin derivative is carried out by treating carboxy group containing porphyrins with a mixture of carbodiimide, N-hydroxy benzothiazole (=HOBT) and N-hydroxy succinimide in organic solvent, usually dimethylformamide. The reactive product formed in this process was used as such, without any purification or isolation of the reactive species. Since in such an activation process a mixture of undefined and unstable products is formed, the isolation of a single reactive species in pure form is very difficult. An additional drawback of the method is that the multiple activated porphyrins formed in the process may cause crosslinking and subsequent inactivation of the bioactive molecules.

Overall, a general problem of the carbodiimide mediated conjugation, when applied to polycarboxy porphyrins, is the lack of selectivity (i.e. all carboxy groups are subject to activation). Another problem is the formation of a complex mixture of labile compounds at the very first chemical stage of the conjugation reaction. For such mixtures it is difficult to apply conventional separation methods, so as to isolate in a pure form individual monofunctional reactive derivative(s), which can be characterised and then used for labelling of biomolecules.

In US 5464741, US 4,707,454 and in EP 0071991 Hendrix describes the use of porphyrins as label. US 5464741 deals with the use of palladium(II) octaethylporphine alpha-isothiocyanate as a phosphorescent label for immunoassays. This monofunctional reagent is highly hydrophobic and not water-soluble. Consequently, it has limited use in labelling of biomolecules. An additional drawback of the reagents described by Hendrix is that the reactive group is bound directly next to the porphyrin moiety without any spacer arm. As usual, the lack of spacer arm has a tendency to significantly reduce the performance of the label. Furthermore, the same confusion with structures and names as found in the patent of Sagner et al., is also present in Hendrix's patent. The chemical structures of the compounds of issue remain ambiguous. Since the compound presented by Hendrix are octaalkyl porphyrins, which are not dealt in this invention, the Hendrix's patent is out of the scope of this invention.

In US 4707545 and EP 0071991 Hendrix describes the use of chlorophyll and porphyrin derivatives as labels for fluoroimmunoassay. The key point in these two patents is the utilisation of the characteristic long Stokes' shift of chlorophyll probes for improving the detection sensitivity of the assay. The coupling of the chlorophyll derivatives to an active assay reagent is carried out by means of in situ activation. No chlorophyll labelling reagent has been isolated. Since these two patents deal only with fluorescent markers, they are out of the scope of present invention.



Shoichet et. al. (Siberian Chem. J. 4, 32, 1991) describes the use of palladium (II) complex of 5-isothiocyanatobenzamino octaethylporphine for labelling of oligonucleotides. This monofunctional labelling reagent is highly hydrophobic and not water-soluble, and consequently has limited use in labelling of biomolecules.

- 5 Braman et. al. (WO 96/11937) describes use of tetraphenylporphyrins for labelling of nucleosides, nucleotides, oligonucleotides and polynucleotides. According to Braman's publication, the porphyrin moiety on the labelled compound is detected on the basis of a reaction product produced by a porphyrin catalysed oxidation reaction. Said oxidation reaction may result in the formation of light, i.e., a
- 10 chemiluminescent reaction, or a coloured compound, i.e., a colorimetric reaction. Since the Braman's publication deals with tetraphenylporphyrins which are not dealt in the present invention, and since detection in Braman's publication is not based on phosphorescence as in the present invention but on colorimetry and chemiluminescence, is the Braman's publication out of the scope of the present
- 15 invention.

#### SUMMARY OF THE INVENTION

- The characteristic features of this invention are disclosed in the claims. The present invention describes novel phosphorescent metalloporphyrin compounds, their preparation and the use of said compounds as labelling reagents for preparation of
- 20 phosphorescent conjugates with biomolecules. The labelling reagents obtainable according to this invention are preferably water soluble monofunctional derivatives of Pt- and Pd-coproporphyrins. The term 'monofunctional' indicates that one of the four originally equal carboxy containing side chains of coproporphyrin has been differentiated and can be selectively further reacted. This invention offers also a
- 25 route for preparation of such labelling reagents as well as the key intermediate compounds in a pure form, in other words, in form of stable organic compounds having defined chemical structure and properties. The compounds can be used as labelling reagents for labelling of biomolecules like peptides, proteins, hormones,

nucleic acids, for labelling of cells, tissue or other chemical species having at least one functional group suitable for labelling reaction. The labelling reaction takes place under mild and slightly alkaline condition and can be carried out by any skilled person in the art. This invention also describes preparation of various  
5 bioconjugates using said metalloporphyrins as labelling reagents and the properties and the use of the such bioconjugates. The invention also describes the use of such bioconjugates in high sensitivity bioaffinity assays based on time-resolved phosphorescence. The bioaffinity assay may be e.g. an immunoassay, nucleic acid hybridisation and it can be performed in solution or on a solid substrate and the  
10 sample can be analysed with a time-resolved fluorometer/phosphorimeter. The assay can also be microscopic investigation of an immunocytological or immunohistological specimen.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The chemistry in the field of the use of the labelling reagents according to this  
15 invention most often takes place in aqueous media. Consequently, an applicable label needs to be water soluble and preferably hydrophilic. However, the aromatic tetrapyrrole unit i.e. porphin found in all porphyrins is hydrophobic in nature and not water soluble. In order to make the porphin unit water soluble, it must be substituted with hydrophilic side groups. One of the most preferable groups for this  
20 purpose is the carboxy group. In this context the term 'carboxy group' means the chemical group that equals with the chemical formula  $-\text{COOH}$ , i.e. the group that corresponds to carboxylic acids. On the other hand, the term 'oxycarbonyl group' means the chemical group that equals with the chemical formula  $-\text{COO}-$ , i.e. the diyl component found in carboxy group as well as in alkoxycarbonyl groups. The  
25 carboxy group is anionic in nature and has a tendency to make otherwise hydrophobic species water soluble in neutral and alkaline pH. Therefore, the most promising porphyrin compounds for labelling of biomolecules are those having carboxy (or the corresponding carboxylate) functional groups as substituents on the porphin periphery. For example, coproporphyrins, which contains carboxy groups,

are sufficiently water-soluble and can be used as a label in aqueous media. In context to this text the term 'coproporphyrin' includes both free bases as well as metallocomplexes of coproporphyrin I and/or II.

- A basic requirement for a commercially valuable labelling reagent is the
- 5 monofunctionality with respect to the number of chemically reactive groups in the labelling reagent. This feature enables conjugation with biomolecules even in high target molecule concentration and high reagent concentration without causing deleterious cross-linking. The use of high concentrations makes labelling process more reliable and produces a product with higher degree of label substitution.
- 10 A further requirement for a commercially valuable labelling reagent is that the method used for the preparation of said reagent must enable reasonably high synthetic yield. An additional requirement is that the method of preparation enables isolation of the labelling reagent in pure form, in other words, in the form of a stable organic compound having a defined chemical structure and defined properties.
- 15 To improve the label performance, it is desirable to have an additional spacer arm (usually 2 to 20 atoms long) between the reactive site and the chromophoric moiety of the reagent. The spacer arm can serve for example to reduce steric hindrance around the reactive site, to make the reactive site more mobile, and to reduce the physical interaction between the label and the biomolecule.
- 20 It can be seen that the phosphorescent porphyrin-based labelling reagents according to prior art, usually lack one or more of the preferred properties.

In present invention we describe novel phosphorescent Pd- and Pt- coproporphyrins which fulfil the preferred properties as discussed above and which can be used for facile labelling of biologically active molecules having one or more functional

25 groups suitable for the conjugation reaction. General structures that are characteristic for the labelling reagents which essentially fulfil the above mentioned requirements, are shown in Scheme 1 (Structure I and Structure II). In these

- compounds **M** is palladium (II) or platinum (II), **R**<sub>1</sub> is -OH, or -O<sup>-</sup>X<sup>+</sup> group where X is a cation, most usually alkali metal cations or ammonium cations and **R**<sub>2</sub> is a chemical moiety bearing a linker unit -Y- and a functional group -Z where said functional -Z group can be used for selective covalent linkage to other molecules, such as biomolecules, and/or for further chemical modifications. The linker unit -Y- is either a covalent bond or a C<sub>1</sub>-C<sub>20</sub> straight or branched alkylene, arylene, alkarylene or aralkylene group, which also may contain heteroatoms or heteroatoms containing side chains, or cyclic residues. The linker unit may also comprise or be composed of residues of polymers, preferably residues of hydrophilic polymers such as residues of polypeptides, polysaccharides, polynucleotides, polyethers or other. Among the most preferable chemical moieties -YZ are, according to this invention, those having a general formula -(CH<sub>2</sub>)<sub>n</sub>-Ph-Z, where -Z is either amino (-NH<sub>2</sub>) or isothiocyanato (-NCS) group, -Ph- is a phenylene group, and n is an integer from 1 to 10.
- From the Structure I and Structure II (Scheme 1) one can see that the main object of this invention is the differentiation and subsequent functionalisation of one single oxycarbonyl group in coproporphyrins used as starting material. Such differentiation is realised via selective hydrolysis of the appropriate tetraalkyl esters of corresponding coproporphyrins, by using simple chemical procedure and technique.
- Although it is clear that simple hydrolysis of tetraesters of coproporphyrins (as well as other polyesters of porphyrins) usually results in number of products, the present invention demonstrates that, if the hydrolysis reaction is carried out under the particular conditions described in this invention, surprisingly high amount of the coproporphyrin monoacid can be produced. After a simple chromatographic separation, a pure monocarboxy product can be isolated, whereas all the other by-products can be pooled and then transformed back into the form of tetraester by simple reesterification. Such reesterification reaction usually proceeds smoothly and almost quantitatively, thus saving most of the raw material which then can be

recycled. Considering easy and simple recycling, the net yield of target monoacid of coproporphyrin may approach its theoretical limit.

The monoacid of coproporphyrin is the key compound for further monofunctionalisation and preparation of labelling reagents and bioconjugates according to this invention. This compound obtained in a pure form can easily be modified into the form of appropriate monofunctionalised derivative(s), labelling reagent(s) or a precursor of a labelling reagent. The modification of the carboxylic acid residue in coproporphyrin monoacid can be performed easily by applying the conventional chemistry related to modification of carboxy groups. The carboxy group of coproporphyrin monoacid can be modified, so as to introduce various functional groups via this site using a variety of available chemical methods .

According to this invention, the preferred methods of monofunctionalisation of the monoacid of coproporphyrins include, for example, activation of the carboxy group for subsequent coupling reactions using methods that involve succinimide esters, sulfosuccinimide esters, pentafluorophenyl esters, 4-nitrophenol esters, N-hydroxy benzotriazole esters, carbodiimide adducts and others.

If further lengthening is desirable, an additional spacer may be attached to the differentiated side chain. This can be carried out by using appropriate compounds such as heterobifunctional linkers. The preferable heterobifunctional compounds according to this invention include, for example, those in which the two functional groups are an aliphatic primary amino group and an aromatic primary amino group. This class of linker compounds is particularly preferred in the scope of this invention because of following reasons. First, due to the remarkable difference in reactivities between the two amino groups, the coupling with an active ester proceeds without a risk of deleterious crosslinking. Second, said class of linker compounds comprise an inherent precursor for the reactive isothiocyanato group. However, the scope of this invention is not limited to the use of said class of linker

compounds, but any of linker compounds, such as homobifunctional linker compounds and polyfunctional linker compounds can also be applied.

The next step in the synthetic scheme of a coproporphyrin labelling reagent is the transformation of relatively hydrophobic triester of the monofunctionalised  
5 coproporphyrin into a water-soluble form by alkaline hydrolysis of the remaining alkoxy carbonyl groups. As described above, the three carboxy substituents provide a structure with solubility in neutral and alkaline aqueous solutions. This stage is expected to preserve the introduced functional group, the spacer and the main structure of the coproporphyrin derivative from undesirable transformations. At this  
10 stage a water-soluble derivative of coproporphyrin is obtained which bears three carboxy groups and one differentiated side chain. The differentiated side chain that comprises a spacer unit and a reactive chemical group, such as amino or hydroxy, can be used for covalent coupling as such or after further chemical modification. For example, a coproporphyrin having an aromatic primary amino group, can be  
15 used for covalent coupling as such, or in the form of its conversion product isothiocyanate, which is obtained from said aromatic primary amino group by treatment with thiophosgene. Corresponding phenyl-isothiocyanato derivatives of phosphorescent Pt- and Pd-coproporphyrins are preferred target compounds and labelling reagents according to this invention. However, the scope of this invention  
20 is not limited to the use of isothiocyanato group as final reactive group, but other chemical groups can also be applied.

In certain cases, where covalent labelling of a target molecule in organic solvent is preferred, the non-water soluble form of a differentiated coproporphyrin, i.e. coproporphyrin comprising up to three alkoxy carbonyl groups, may also be applied  
25 as a labelling reagent. Scheme 1 (Structure I and Structure II) can also be used to illustrate the general structures of such compounds. In this case M is palladium (II) or platinum (II), R<sub>1</sub> is -OR group where R is C<sub>1</sub>- C<sub>20</sub> straight or branched alkyl, aryl, alkaryl or aralkyl group, R<sub>2</sub> is a chemical moiety bearing a linker unit -Y- and a functional group -Z where said functional -Z group can be used for selective

covalent linkage to other molecules, such as biomolecules, and/or for further chemical modifications. The linker unit -Y- is either a covalent bond or a C<sub>1</sub>-C<sub>20</sub> straight or branched alkylene, arylene, alkarylene or aralkylene group, which also may contain heteroatoms or heteroatoms containing sidechains, or cyclic residues.

- 5 The linker unit may also comprise or be composed of residues of polymers, preferably residues of hydrophilic polymers such as residues of polypeptides, polysaccharides, polynucleotides, polyethers or other. Among the most preferable chemical moieties -YZ are, according to this invention, those having a general formula  $-(CH_2)_n-Ph-Z$ , where -Z is either amino (-NH<sub>2</sub>) or isothiocyanato (-NCS) group, -Ph- is a phenylene group, and n is an integer from 1 to 10.

The phosphorescent labelling reagents described above such as water-soluble phenyl-isothiocyanate derivatives of Pt- and Pd-coproporphyrins, can be used for facile labelling of various biomolecules such as peptides, proteins, haptens, nucleic acids; labelling of cells, tissue or other chemical species. As products of such labelling reactions the phosphorescent conjugates can be obtained, which are also the object of this invention. Said conjugates can be used as probes in sensitive bioaffinity assays, which are based on bioaffinity reactions and time-resolved phosphorescence detection.

The following non-limiting Examples are aimed to further demonstrate the invention. In the Examples below the compounds referred to (Compound 1 etc.) are disclosed in Schemes 2 to 8.

#### EXAMPLE 1. Synthesis of coproporphyrin I tetraisoamyl ester (Compound 2).

A mixture of coproporphyrin I dihydrochloride (Compound 1, 300 mg, 412 µmol, prepared according to Smith et al., J.Chem. Soc. Perkin Trans I, 1471-1475 (1972)), 10 mL of isoamylalcohol and 0.5 mL of concentrated sulfuric acid was stirred at room temperature for 72 h. Then 1 mL of triethylamine was added, and the mixture was evaporated to dryness under reduced pressure at 100 °C. The residue was washed with water and the organic phase was dried by filtration through anhydrous

sodium sulphate to give 351 mg (91%) of the corresponding tetraester (Compound 2).

EXAMPLE 2. Synthesis of coproporphyrin I tetraethyl ester (Compound 3).

To a mixture of of coproporphyrin I dihydrochloride (Compound 1, 300 mg, 412  
5  $\mu\text{mol}$ ) and 50 mL of absolute ethanol, 2.5 mL of concentrated sulfuric acid was  
added and the mixture was stirred for 48 h at room temperature. Then 25 % aqueous  
solution of ammonia was added until pH 4. The precipitate was filtered off, washed  
with 50 mL of water and dried under atmosphere. The product was crystallised from  
methylene chloride / methanol and dried under atmosphere to give 290 mg (92%) of  
10 Compound 3.

EXAMPLE 3. Synthesis of coproporphyrin I triethyl ester (Compound 5).

To the mixture of Compound 3 (325 mg, 423  $\mu\text{mol}$ ) and 70 mL of tetrahydrofuran,  
70 mL of 6 M hydrochloric acid was added and the reaction mixture was stirred for  
10 minutes at room temperature. The reaction was stopped by addition of trisodium  
15 citrate (1 M, aq ) until pH 4. The organic phase of the two phase system was diluted  
with 80 mL of chloroform, washed with water (50 mL), dried with anhydrous  
sodium sulphate (10 g) and evaporated to dryness. The products of hydrolysis were  
separated by column chromatography on silica gel using a stepwise gradient of  
methanol (0% -20%) in chloroform, as eluent. The first eluting compound was the  
20 recovered starting material (Compound 3), yield 104 mg (32%). The second eluting  
compound was the desired product Compound 5, yield 115 mg (37%).

EXAMPLE 4. Synthesis of coproporphyrin II triethyl ester (Compound 7).

To the mixture of (390 mg, 509  $\mu\text{mol}$ ) of coproporphyrin II tetraethyl ester  
(Compound 6, prepared according to Clezy et al., Aust. J. Chem., 37, 143-154  
25 (1984)) and 100 mL of tetrahydrofuran, 100 mL of hydrochloric acid (6 M, aq) was  
added and the reaction mixture was stirred for 10 minutes at room temperature. The  
reaction was stopped by addition of trisodium citrate (1 M, aq) until pH 4. The



organic phase of the two phase system was diluted with 100 mL of chloroform, washed with water (50 mL), dried with anhydrous sodium sulphate and evaporated to dryness. The products of hydrolysis were separated by column chromatography on silica gel using a stepwise gradient of methanol (0% -20%) in chloroform, as eluent. The first eluting compound was the recovered starting material (Compound 6), yield 164 mg (42%). The second eluting compound was the desired product (Compound 7), yield 115 mg (31%).

EXAMPLE 5. Synthesis of Pd-coproporphyrin II triethyl ester (Compound 8).

Mixture of 63 mg of PdCl<sub>2</sub> and 1 mL of N,N-dimethyl formamide (later DMF) was stirred for 5 minutes at 110 °C followed by decantation of the resulting brownish PdCl<sub>2</sub> solution. To the solid PdCl<sub>2</sub> residue an additional 1 mL portion of DMF was added and the solution was stirred at 110 °C for 5 minutes and decanted. The DMF treatment was repeated one more time and then all the three DMF fractions were combined. The DMF solution was heated to 110 °C and then added to hot DMF (5 mL) solution of Compound 7 (64 mg). The mixture was stirred at 110 °C until the characteristic absorption band for freebase CPII (Compound 7) at 620 nm disappears. Water (5 mL) was dropwise added to the hot reaction mixture and the precipitated product was filtered off. The precipitate was washed with water (5 mL), with ethanol (absolute, 5 ml) and dried under atmosphere. The product was dissolved in chloroform (20 mL) and filtered through a 2 cm thick layer of silica gel using CHCl<sub>3</sub>-MeOH, 9:1 v/v, as an additional eluent. The filtrate was concentrated under reduced pressure and the product was crystallised from chloroform / methanol. The crystals were filtered off and dried under atmosphere to give 65 mg (90%) of Compound 8.

25

EXAMPLE 6. Synthesis of Pt-coproporphyrin I tetraethyl ester (Compound 9).

To a solution of Compound 3 (300 mg, 391  $\mu\text{mol}$ ) in 10 mL of benzonitrile,  $\text{PtCl}_2$ , (600 mg, 2.26 mmol, 5.77 eq.) was added. The mixture was kept at 200 °C for 30 minutes. The cooled mixture was evaporated to dryness under reduced pressure at 150 °C. The residue was purified by filtration through silica gel using chloroform as an eluent. The yield of Compound 9 was 286 mg (76%).

EXAMPLE 7. Synthesis of coproporphyrin I triisoamyl ester (Compound 4).

To a stirred solution of Compound 2 (290 mg, 310  $\mu\text{mol}$ ) in 1,4-dioxane (50 mL), hydrochloric acid (50 mL, 6M, aq.) was added. The mixture was stirred at room temperature for 20 min, diluted with chloroform (100 mL), washed with water, aqueous ammonia, dried with anhydrous sodium sulphate and evaporated to dryness under reduced pressure at 40 °C. The residue was purified by column chromatography on silica gel with chloroform - methanol (30:1) to give 120 mg (41%) of starting Compound 2 and 132 mg (49%) of mono acid derivative, Compound 4.

EXAMPLE 8. Synthesis of Pd-coproporphyrin I triisoamyl ester (Compound 10).

Compound 4 (132 mg, 49%) was dissolved in of N,N-dimethylformamide (5 mL), heated to 100 °C and a solution of  $\text{PdCl}_2$  (130 mg, 733  $\mu\text{mol}$ ) in of of N,N-dimethylformamide (2 mL) was added. The mixture was kept at 100 °C for 20 min. To the cooled mixture 0.2 mL of water was added. The precipitate was filtered off, washed with water and dried under atmosphere. The product was purified by filtration through silica gel with chloroform - methanol (20:1) as an eluent to give 135 mg (91%) of Compound 10.

EXAMPLE 9. Synthesis of Compound 11, Pt-coproporphyrin I triethyl ester.

To a refluxing solution of Compound 9 (220 mg, 229  $\mu\text{mol}$ ) in 1,4-dioxane (70 mL), hydrochloric acid (2.1 mL, 6M, aq.) was added. The mixture was refluxed for 20

min, cooled, diluted with chloroform (100 mL), washed with water, aqueous ammonia, and evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel using chloroform - methanol (30:1) as an eluent to give 110 mg (50%) of starting material (Compound 9) and 75 mg (35%) of the mono acid derivative (Compound 11).

EXAMPLE 10. Synthesis of Compound 13, a derivative of Pt-coproporphyrin I triethyl ester having 2-(4-aminophenyl)ethylamino side chain.

Compound 11 (75 mg, 80.5  $\mu$ mol) was dissolved in methylene chloride (7 mL), and triethylamine (0.2 mL, 1.42 mmol) was added followed by addition of pentafluorophenyl trifluoroacetate (30  $\mu$ L, 175  $\mu$ mol). The mixture was stirred at room temperature for 10 min (Compound 12 formation) and 2-(4-aminophenyl)ethylamine (25 mg, 184  $\mu$ mol) was added. The mixture was stirred at room temperature for 10 min, diluted with chloroform (20 mL), washed with water, hydrochloric acid (1M, aq.), water, sodium bicarbonate (saturated aqueous solution), dried with anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica gel using chloroform - methanol (30:1) as an eluent to give 63 mg (75%) of Compound 13.  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ , 400 MHz): 9.40, 9.39, 9.32 and 9.31 (s, 4 H, meso-H), 6.19 and 5.93 (d, 4 H,  $J = 8.4$  Hz, AB-system of Ph), 5.31 (t, 1 H,  $J = 5.6$  Hz,  $\text{CONHCH}_2$ ), 4.22 - 4.15 (m, 6 H,  $\text{COOCH}_2\text{CH}_3$ ), 4.02 (m, 8 H,  $\text{CH}_2\text{CH}_2\text{COOR}$ (3) and  $\text{CH}_2\text{CH}_2\text{CONH}$ ), 3.31, 3.263, 3.26 and 3.25 (s, 12 H, peripheral  $\text{CH}_3$ ), 3.17 (m, 2 H,  $\text{CONHCH}_2\text{CH}_2\text{PhNH}_2$ ), 3.05 (m, 6 H,  $\text{CH}_2\text{CH}_2\text{COOR}$ ), 2.83 (t, 2 H,  $J = 7.1$  Hz,  $\text{CH}_2\text{CH}_2\text{CONH}$ ), 2.08 (t, 2 H,  $J = 6.8$  Hz,  $\text{CONHCH}_2\text{CH}_2\text{PhNH}_2$ ), 1.20 (m, 6 H,  $\text{COOCH}_2\text{CH}_3$ ).

EXAMPLE 11. Synthesis of Compound 15, a derivative of Pd-coproporphyrin I triisoamyl ester having 2-(4-aminophenyl)ethylamino side chain.

To a stirred solution of Compound 10 (210 mg, 216.6  $\mu$ mol) and triethylamine (0.3 mL, 2.13 mmol) in methylene chloride (15 mL), pentafluorophenyl trifluoroacetate

(60  $\mu$ L, 349  $\mu$ mol) was added. The mixture was stirred at room temperature for 10 min (formation of Compound 14), and 2-(4-aminophenyl)ethylamine (48 mg, 350  $\mu$ mol) was added. The mixture was stirred at room temperature for 10 min, diluted with chloroform (50 mL), washed with water, hydrochloric acid (1M, aq.), water, sodium bicarbonate (saturated aqueous solution), dried with anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The product was purified by column chromatography on silica gel using chloroform - methanol (30:1) as an eluent to give 208 mg (88%) of Compound 15.  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ , 400 MHz): 10.03, 10.01, and 10.00(2) (s, 4 H, meso-H), 6.05 and 5.79 (d, 4 H,  $J = 8.2$  Hz, AB-system of Ph), 5.08 (m, 1 H,  $\text{CONHCH}_2$ ), 4.33 (m, 8 H,  $\text{CH}_2\text{CH}_2\text{COOR}$ (3) and  $\text{CH}_2\text{CH}_2\text{CONH}$ ), 4.17 (t, 6 H,  $J = 6.8$  Hz,  $\text{COOCH}_2\text{CH}_2$ -), 4.03 and 3.96 (m, 3 H,  $\text{OCH}_2\text{CH}_2\text{CH}(\text{CH}_3)_2$ ), 3.60, 3.58(2), and 3.56 (s, 12 H, peripheral  $\text{CH}_3$ ), 3.22 (m, 8 H,  $\text{CH}_2\text{CH}_2\text{COOR}$ (3) and  $\text{CONHCH}_2\text{CH}_2\text{PhNH}_2$ ), 3.01 (t, 2 H,  $J = 7.1$  Hz,  $\text{CH}_2\text{CH}_2\text{CONH}$ ), 2.04 (t, 2 H,  $J = 6.8$  Hz,  $\text{CONHCH}_2\text{CH}_2\text{PhNH}_2$ ), 1.44 (m, 6 H,  $\text{OCH}_2\text{CH}_2\text{CH}(\text{CH}_3)_2$ ), 0.79 (m, 18 H,  $\text{OCH}_2\text{CH}_2\text{CH}(\text{CH}_3)_2$ ).

EXAMPLE 12. Synthesis of Compound 19, a derivative of Pd-coproporphyrin I triethyl ester having 3-aminopropylamino side chain.

To a stirred solution of Compound 16 (34.5 mg, 40.9  $\mu$ mol) and triethylamine (41 mg, 409  $\mu$ mol) in methylene chloride (2.5 mL) pentafluorophenyl trifluoroacetate (34.4 mg, 123  $\mu$ mol) was added (formation of Compound 17). The solution was stirred for 60 minutes at room temperature and then mono-N-(tBOC)-propylenediamine (28.2 mg, 164  $\mu$ mol) was added. The reaction mixture was stirred overnight at room temperature. Chloroform (10 mL) and sodium carbonate (10 mL, 1 M, aq.) were added and the mixture was stirred for 15 minutes. The organic phase was separated and washed with water (10 mL), citric acid (10 mL, 10% aq.) and finally with water (10 mL). The solution was dried with anhydrous sodium sulphate and evaporated to dryness and to give 33.5 mg (82%) of Compound 18. The product was dissolved in 6 mL of chloroform and 2 mL of trifluoroacetic acid was added.

The solution was stirred for 20 minutes at room temperature and evaporated to dryness under reduced pressure. The residue was dissolved in chloroform (10 mL) and washed with sodium carbonate (10 mL, 1 M, aq.) and water (10 mL). The solution was dried with anhydrous sodium sulphate and concentrated to 1 mL. The product was precipitated by dropwise addition of petroleum ether, filtered off and dried under atmosphere. Yield of Compound 19 was 27,0 mg (72%).

EXAMPLE 13. Synthesis of Compound 20, a derivative of Pd-coproporphyrin I triethyl ester having 2-aminoethylamino side chain

A solution of Compound 17 (25 mg, 24.8  $\mu$ mol) in methylene chloride (5 mL) was added to a solution of ethylenediamine (50  $\mu$ L) in methylene chloride (10 mL). The mixture was stirred at room temperature for 30 min, washed with water (30 mL), dried with anhydrous sodium sulphate, and evaporated to dryness to give 20 mg (92%) of Compound 20.

EXAMPLE 14. Synthesis of Compound 22, a derivative of Pd-coproporphyrin II triethyl ester having 2-(4-aminophenyl)ethylamino side chain.

To a solution of Compound 8 (28.5 mg, 33,8  $\mu$ mol) and triethylamine (20  $\mu$ L, 144  $\mu$ mol) in methylene chloride (5 mL), pentafluorophenyl trifluoroacetate (40  $\mu$ L, 142  $\mu$ mol) was added. After stirring the solution for 15 minutes at room temperature 2-(4-aminophenyl)ethylamine (35 mg, 181  $\mu$ mol) was added and the solution was stirred further for 1 h. The solution was diluted with methylene chloride (10 mL), washed with water (15 mL), hydrochloric acid (15 mL, 1 M, aq.), water (15 mL), sodium bicarbonate (15 mL, 1 M, aq.), dried with anhydrous sodium sulphate and evaporated to dryness. The product was crystallised from methylene chloride / methanol, filtered off and dried under atmosphere to give 29,1 mg (90 %) of Compound 22.

EXAMPLE 15. Synthesis of Compound 24, N-(2-(4-aminophenyl)ethyl)-2-aminoethanamide.

To a solution of tBoc-glycine (1.36 g, 7.77 mmol) and N-hydroxysuccinimide (894 mg, 7.77 mmol) in dioxane (3 mL), solution of dicyclohexyl carbodiimide (1.76 g, 8.47 mmol) in dioxane (3 mL) was added. After stirring the solution for 3 hours at room temperature the urea precipitate was filtrated off and the solution was

5 evaporated to dryness. The product was crytallised from chloroform to give 950 mg (45 %) of Compound 23a. Compound 23a (360 mg, 1.32 mmol) was dissolved in acetone and 2-(4-aminophenyl)ethylamine (216 mg, 1.58 mmol) was added. After stirring for 15 minutes at room temperature the solution was evaporated to dryness, dissolved in chloroform (10 mL) , washed with water (2x15ml), dried with

10 anhydrous sodium sulphate and evaporated to dryness. The residue was further purified by column chromatography on silica gel using 0-20% methanol / chloroform as eluent: The yield of Compound 23b was 329 mg (85 %). Compound 23b (329 mg, 1.12 mmol) was dissolved in chloroform (8 mL) and trifluoroacetic acid (4 mL) was added. After stirring for 30 minutes at room temperature the

15 solution was evaporated to dryness. The residue was dissolved in water (6 mL) and the solution was made alkaline by addition of sodium hydroxide (6M, aq.) The aqueous solution was extracted with chloroform (6x 30 mL). The chloroform fractions were combined and dried with anhydrous sodium sulphate. The solution was evaporated to dryness to give 175 mg (81%) of N-(2-(4-aminophenyl)ethyl)-2-aminoethanamide (Compound 24). Compound 25 (N-(2-(4-aminophenyl)ethyl)-2-

20 aminopropanamide was prepared according to an analogous procedure as for N-(2-(4-aminophenyl)ethyl)-2-aminoethanamide.

EXAMPLE 16. Synthesis of Compound 26, a derivative of Pd coproporphyrin I triethyl ester having a 2-(4-aminophenyl)ethylcarbamylmethylamino side chain.

25 To a stirred solution of Compound 16 (71 mg, 84  $\mu$ mol) and triethyl amine (84.8 mg, 840  $\mu$ mol) in methylene chloride (5 mL), pentafluorophenyl trifluoroacetate (94.0 mg, 336  $\mu$ mol) was added and the solution was stirred for 30 minutes at room temperature (formation of Compound 17). N-(2-(4-aminophenyl)ethyl)-2-aminoethanamide (Compound 24)(65 mg, 336  $\mu$ mol) was added and the solution

was stirred overnight at room temperature. The solution was diluted with methylene chloride (10 mL) and washed with sodium carbonate (10 mL, 1M, aq.), water (10 mL), citric acid (10 mL, 10%, aq.) and with water (10 mL). The solution was dried with anhydrous sodium sulphate, concentrated to volume of 2 ml and the product  
5 was precipitated by addition of hexane. The product was separated by centrifugation and dried under vacuum to give 65 mg (76%) of Compound 26.

EXAMPLE 17. Synthesis of Compound 27, a derivative of Pd coproporphyrin I having a 2-(4-aminophenyl)ethylcarbamylmethylamino side chain.

To refluxing solution Compound 26 (65 mg) in dioxane (5 mL), sodium hydroxide  
10 (200  $\mu$ l, 6 M, aq.) was added. The solution was refluxed for 15 minutes, 5 mL of water was added and refluxing was continued for 30 minutes. The cooled solution was washed with chloroform and 10% solution of citric acid was added until pH 4. The precipitate was separated by centrifugation and the pellet was washed twice with water (resuspending and centrifuging) and evaporated to dryness twice from  
15 absolute EtOH. The product was further dried in desiccator over  $P_2O_5$  to give 50 mg (84%) of Compound 27.

EXAMPLE 18. Synthesis of Compound 28, a derivative of Pd-coproporphyrin I triethyl ester having 2-(4-aminophenyl)ethylcarbamylethylamino side chain.

To a solution of Compound 16 (39.5 mg, 46,8  $\mu$ mol ) and triethyl amine (47.3 mg, 468  $\mu$ mol) in methylene chloride (3 ml), a solution of pentafluorophenyl  
20 trifluoroacetate (59.0 mg, 211  $\mu$ mol ) was added (formation of Compound 17). Compound 25 (87.4 mg, 422  $\mu$ mol) (for preparation see EXAMPLE 13) in DMF (1 mL) was added to the reaction mixture and the mixture was stirred overnight at room temperature. The solution was diluted with chloroform (15 ml), washed with  
25 sodium carbonate (1 M, aq.), water (15 ml), citric acid (15 ml, 10 %), water (15 ml) and dried with anhydrous sodium sulphate. The product was evaporated to dryness and purified by preparative TLC using 10% methanol - chloroform as an eluent to give 31.4 mg (65 %) of Compound 28.

EXAMPLE 19. Synthesis of Compound 29, a derivative of Pd-coproporphyrin I triethyl ester having a  $\gamma$ -(N-maleimido) butylamidopropylamino side chain.

To a solution of Compound 19 (13.0 mg, 12.8  $\mu$ mol) in chloroform (1 mL), triethylamine (12.9 mg, 128  $\mu$ mol) and  $\gamma$ -(N-maleimido)butyroxyl succinimide (7.2 mg, 25.6  $\mu$ mol) were added and the solution was stirred overnight at room temperature. Sodium bicarbonate (1 mL, saturated aqueous solution) was added and the mixture was stirred for 1 h. The mixture was diluted with chloroform (5 mL) and the organic phase was washed two times with 10 mL of water, dried with sodium sulphate and evaporated to dryness. The residue was dissolved in methylene chloride (1 mL) and precipitated by dropwise addition of hexane. The precipitate was filtered off and dried under atmosphere. Yield of Compound 29 was 13.2 mg (97%).

EXAMPLE 20. Synthesis of Compound 30, a derivative of Pd-coproporphyrin I having 2-aminoethylamino side chain

To a refluxing solution of Compound 20 (20 mg, 22.6  $\mu$ mol) in dioxane (15 mL) sodium hydroxide (0.2 mL, 6M aq.) was added. The mixture was refluxed for 30 min, 15 mL of water was added, refluxed for additional 30 min, cooled, and washed with methylene chloride (30 mL). To the resulting aqueous phase hydrochloric acid (1M) was added until pH 4. The precipitate was separated by centrifugation, washed with water (2x10 mL), and dried at 40 °C under vacuum (~20 mmHg) to yield 17 mg (90%) of Compound 30.

EXAMPLE 21. Synthesis of Compound 31, a derivative of Pd-coproporphyrin I having 3-aminopropylamino side chain

To a refluxing solution of Compound 19 (25 mg, 27.3  $\mu$ mol) in dioxane (10 mL), sodium hydroxide (200  $\mu$ L, 6 M, aq.) was added and refluxing was continued for 20 minutes. 10 mL of water was added and refluxing was continued for 30 minutes. The cooled solution was washed with chloroform (10 mL) and the product was



precipitated by addition of hydrochloric acid (1 M) until pH 4. The precipitate was separated by centrifugation, washed with water (2x10 mL) and evaporated twice to dryness from absolute ethanol. The product was further dried in desiccator over silica gel to give 19 mg (80 %) of Compound 31.

- 5    **EXAMPLE 22.** Synthesis of Compound 32, a derivative of Pd-coproporphyrin I having 4-(N-maleimidomethyl)cyclohexylcarboxamidoethylamino side chain.

To a stirred suspension of 12 mg (14.3  $\mu$ mol) of Compound 30 in water (10 mL) sodium bicarbonate (40 mg) was added and clear solution was obtained after stirring at 40 °C for 10 min. The solution was diluted with dioxane (10 mL) and N-succinimidyl 4-(maleimidomethyl)cyclohexane carboxylate (20 mg) in dioxane (2 mL) was added. The mixture was stirred at room temperature for 12 h. The reaction mixture was washed with methylene chloride (30 mL), and hydrochloric acid (1M, aq.) was added until pH 4. The precipitate was separated by centrifugation, washed with water (2x10 mL), and dried at 40 °C in vacuum (~20 mm Hg) to yield 13 mg  
10  
15 (90%) of Compound 32.

**EXAMPLE 23 .** Synthesis of compound 34, a derivative of Pd-coproporphyrin I having 2-(4-isothiocyanatophenyl)ethylamino side chain.

To a refluxing solution of Compound 21 (100 mg, 91.9  $\mu$ mol) in dioxane (35 mL), sodium hydroxide (0.2 mL, 6M aq) was added. The mixture was refluxed for 30  
20 min, water (20 mL) was added and the mixture was refluxed for additional 30 min. The reaction mixture was washed with chloroform (30 ml), and then chloroform (20 mL) and sodium bicarbonate (5 mL, saturated aqueous solution) were added. To the vigorously stirred mixture, thiophosgene (90  $\mu$ l, 1.17  $\mu$ mol) was added and the stirring was continued at room temperature for 30 min. The layers were separated  
25 and the aqueous phase was washed with chloroform. The product was precipitated by addition of hydrochloric acid (2M, aq.) until pH 4. The precipitate was separated

by centrifugation, washed with water, and dried in vacuum desiccator over silica gel to yield 65 mg (77%) of Compound 34.

EXAMPLE 24. Synthesis of Compound 35, a derivative of Pd-coproporphyrin I  
5 triethyl ester having 6-(1-O-methyl)mannopyranosylamino side chain.

To a solution of Compound 17 (6 mg, 5.9  $\mu$ mol) in methylene chloride (2 mL) a solution of 6-amino-2,3-O-isopropylidene-methyl  $\alpha$ -D-mannopyranoside (10 mg) in 1 mL of pyridine was added. The mixture was stirred at room temperature for 30 minutes, washed with water (30 mL), dried with anhydrous sodium sulphate, and  
10 concentrated under reduced pressure. The product was purified by column chromatography on silica gel (20 mL) using diethyl ether - methylene chloride (10:90) as an eluent to give Compound 35, which was precipitated from methylene chloride (1 mL) by addition of hexane (5 mL). The precipitate was filtered off and dried at 40 °C under vacuum (~20 mmHg) to give 6 mg (89%) of Compound 35. <sup>1</sup>H-  
15 NMR (CDCl<sub>3</sub>, 300 MHz): 9.87, 9.85, 9.81 and 9.80 (s, 4 H, meso-H), 5.85 (dd, 1 H, J = 3.9, 7.8 Hz, CONH), 4.15-4.35 (m, 14 H, COOCH<sub>2</sub>CH<sub>3</sub>, CH<sub>2</sub>CH<sub>2</sub>COOR(3) and CH<sub>2</sub>CH<sub>2</sub>CONH), 3.54, 3.53, 3.50 and 3.49 (s, 12 H, peripheral CH<sub>3</sub>), 3.20 (m, 8 H, CH<sub>2</sub>CH<sub>2</sub>COOR), 3.06 (s, 3 H, OMe from sugar) 2.70 (m, 2 H, NHCH<sub>2</sub>), 1.40 (s, 6 H, Me of acetonide), 1.20 (m, 9 H, OCH<sub>2</sub>CH<sub>3</sub>).

20 EXAMPLE 25. Synthesis of Compound 36, a derivative of Pd-coproporphyrin I having 6-(1-O-methyl)mannopyranosylamino side chain.

To a refluxing solution of Compound 35 (6 mg, 5.2  $\mu$ mol) in dioxane (4 mL), hydrochloric acid (0.1 mL, 6M, aq.) was added. The mixture was refluxed for 10 min and 0.2 mL of 6M aqueous solution of sodium hydroxide was added. The  
25 mixture was refluxed for 30 min, 5 mL of water was added, the mixture was refluxed additional 30 min, cooled, and washed with methylene chloride (10 mL). To the resulting aqueous phase, hydrochloric acid (1M, aq.) was added until pH 4.

The precipitate was separated by centrifugation, washed with water (2x10 mL), and dried at 40 °C under vacuum (~20 mmHg) to yield 4.5 mg (85%) of Compound 36.

EXAMPLE 26. Synthesis of Compound 38, a derivative of Pt-coproporphyrin I  
5 having 2-(4-isothiocyanatophenyl)ethylamino side chain.

To a refluxing solution of Compound 13 (57 mg, 54.3  $\mu$ mol) in dioxane (20 mL), sodium hydroxide (0.2 mL, 6M, aq.) was added. The mixture was refluxed for 30 min, 10 mL of water was added, the mixture was refluxed for additional 30 min, cooled, and washed with chloroform. To the resulting aqueous solution of trisodium  
10 salt of Compound 37, chloroform (20 mL) and sodium bicarbonate (5 mL, saturated solution) were added followed by addition of thiophosgene (60  $\mu$ L, 780  $\mu$ mol). The mixture was vigorously stirred at room temperature for 30 min, and the layers were separated. The aqueous phase was washed with chloroform, and the product was precipitated by addition of hydrochloric acid (2M, aq.) until pH 4. The precipitate  
15 was separated by centrifugation, washed with water, and dried in vacuum desiccator over silica gel to yield 23 mg (48%) of Compound 38.

EXAMPLE 27. Labelling of Rabbit anti-mouse IgG with Compound 34

1 mg of Compound 34 was dissolved in 0.5 mL of dimethylformamide and 0.05 mL of this solution was added to 1 mL of Rabbit anti-mouse-IgG antibody solution  
20 (1 mg/mL, 0.05 M carbonate buffer, pH 9.5). The mixture was incubated overnight at room temperature and then passed through a Sephadex G-25 column equilibrated with PBS buffer (phosphate 50 mM, NaCl 150 mM, pH 7.5). The fraction of the first peak which corresponds to the labelled protein was collected. Labelled IgG was characterised spectrophotometrically and then stored at +4 °C in presence of 1%  
25 BSA and 0.1% sodium azide, for further use.

EXAMPLE 28. Mouse IgG immunoassay using a biologically active assay reagent labelled with Compound 38.

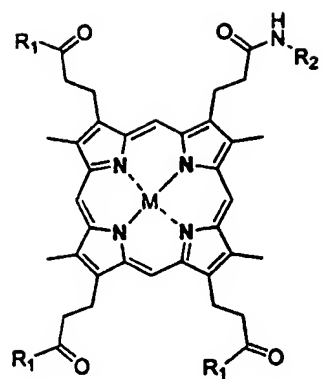
12-well microtiter strips (Nunc) were coated with polyclonal goat anti-mouse-IgG antibody. 0.2 mL of antibody solution (10 microgram/mL) in carbonate buffer, pH 9.6, were added to each well and incubated overnight at room temperature. To inhibit non-specific binding the strips were post-coated with PBS containing bovine serum albumine (bovine serum albumine 10 mg/mL, phosphate 50 mM, NaCl 150 mM, pH 7.5, 1h, room temperature). The strips were washed with PBS containing 0.05% (w/w) of Tween-20 (PBST).

Serial dilutions of mouse IgG were made in PBST, 0.2 mL of each concentration was added to the wells of microtiter strip, incubated for 1h at 37°C and washed 4 times with PBST. Then 0.1 mL of anti-mouse-IgG antibody labelled with Compound 38 (1 microgram/mL) was added to each well, incubated for 1 h at 37°C and then washed 4 times with PBST. The anti-mouse-IgG conjugate was prepared by analogous manner to Example 27 but using Compound 38 and Mouse IgG instead of 34 and Rabbit IgG.

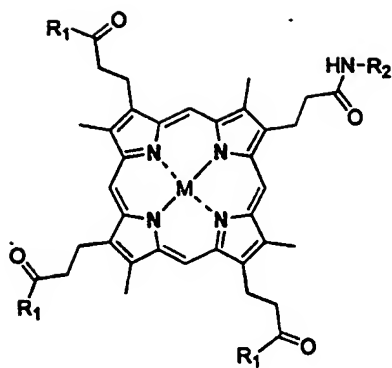
A solution of 0.01 M KOH containing 3 mM cetyltrimethylammonium bromide was added to each well (0.2 mL) and incubated 15 min to desorb labelled antibodies from the solid phase. Then 0.02 mL of freshly prepared solution containing 50 mg/mL of  $\text{Na}_2\text{SO}_3$ , 60 mg/mL  $\text{KH}_2\text{PO}_4$  was added into each well and time-resolved phosphorescence was counted using time-resolved fluorescent reader Arcus-1230 (trademark of Wallac Oy, Turku, Finland). The Arcus was equipped with 535 nm excitation and 650 nm emission filters which correspond to bands of the dye. The delay time was set to 0.05 ms, whereas the gate time was 0.2 ms. Measured signals were plotted as a function of concentration of mouse IgG (i.e. antigen) to obtain a calibration graph.

EXAMPLE 29. Immunocytochemical staining of human colon cancer cells

Detection of cancer antigen C242 in isolated human colon cancer cell line (C205) was used as model for investigation the feasibility of Compound 34 for in-situ immunostaining. The cells were grown in plastic Petri dishes in RPMI-1640 culture medium for 24 hours at 38°C. For specific labelling, the cultured cells were rinsed  
5 once with PBS-BSA (0.1% bovine serum albumin and 0.9 % NaCl in phosphate buffer, pH 7.4) and incubated with a monoclonal anti-human C242 antibody (Wallac, Turku, Finland) at a concentration of 10 µg/mL in PBS for 30 min at room temperature. The cells were then rinsed three times for 5 min with PBS-BSA. After removing the cells from the Petri dish by scraping, they were incubated with Rabbit  
10 anti mouse IgG labelled with Compound 34, at a concentration of 10 µg/mL for 30 minutes. The cells were rinsed with PBS, fixed with 3 % glutaraldehyde (Fluka) in PBS for 10 min at room temperature and rinsed with PBS. The cells were dehydrated through increasing alcohol concentrations, cleared in xylene in a Coplin jar and mounted in Merckoglas (trademark of Merck, Germany). Imaging was  
15 carried out using the Leitz Aristoplan fluorescence microscope equipped with appropriate excitation and emission filters for time-resolved fluorescence imaging as described by Sevéus et al. in Cytometry, 13 (1992) pp 329-338 and in WO 9410568.



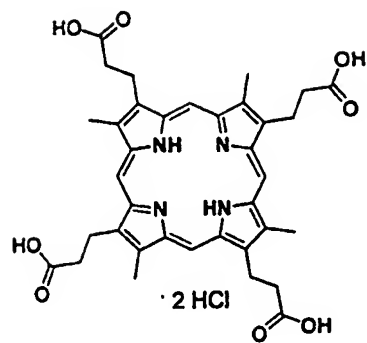
Structure I



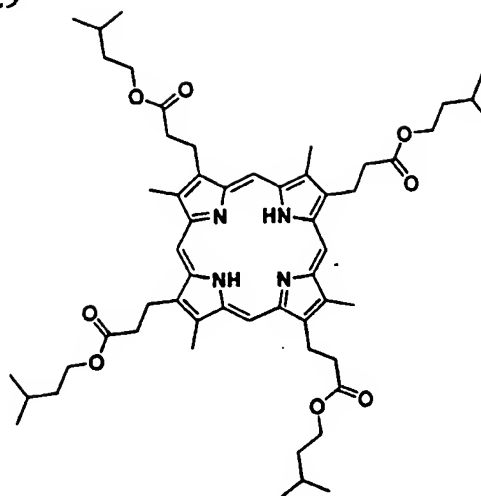
Structure II

Scheme 1

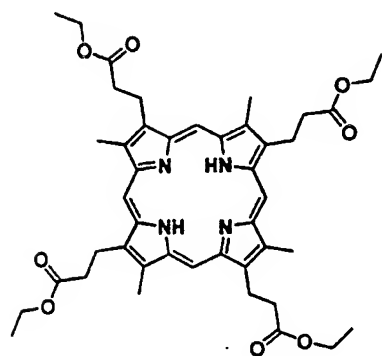
29



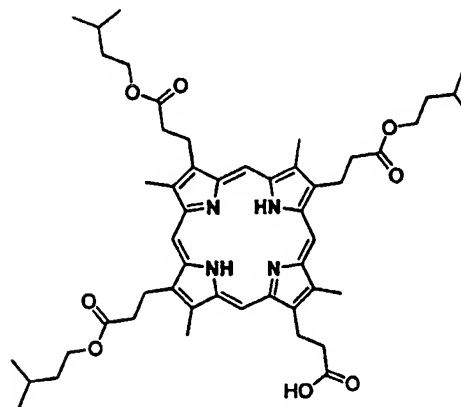
Compound 1



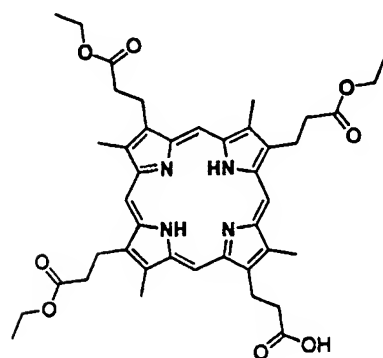
Compound 2



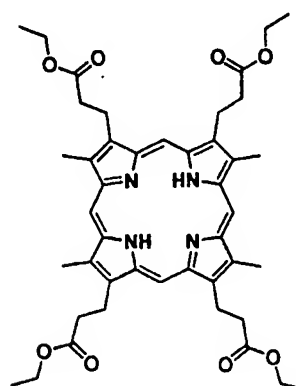
Compound 3



Compound 4



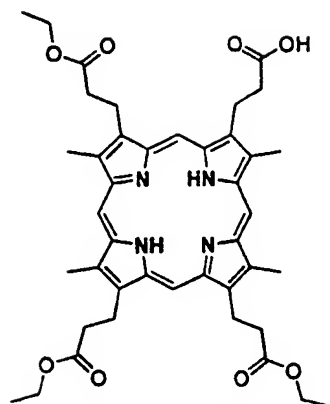
Compound 5



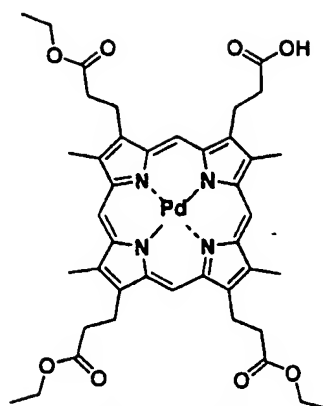
Compound 6

Scheme 2

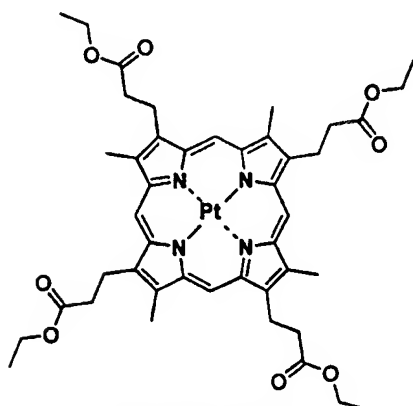
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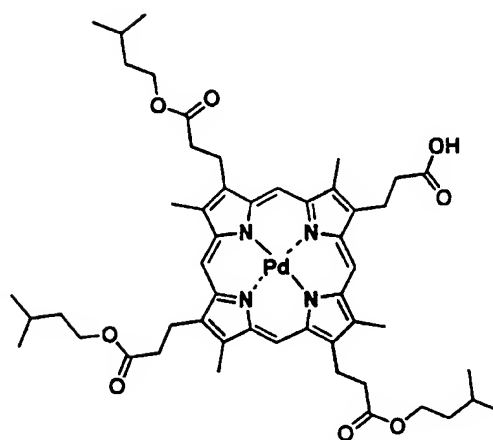
Compound 7



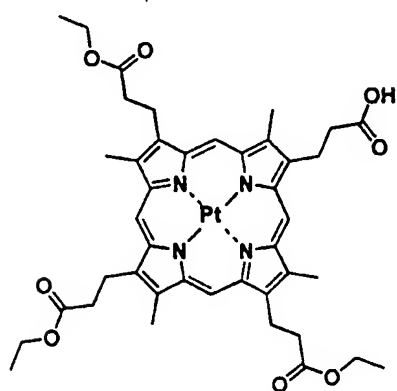
Compound 8



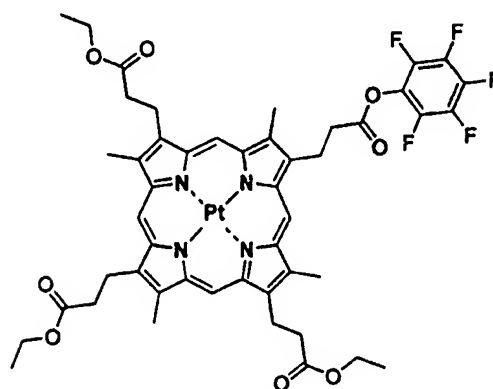
Compound 9



Compound 10



Compound 11

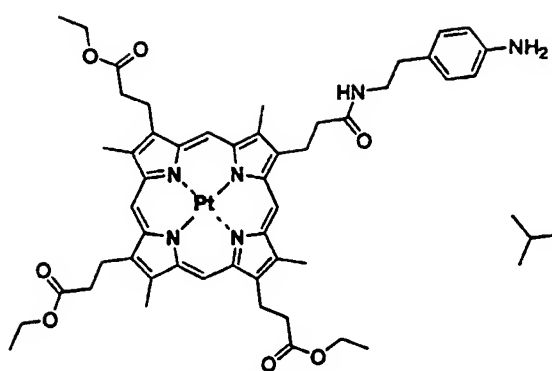


Compound 12

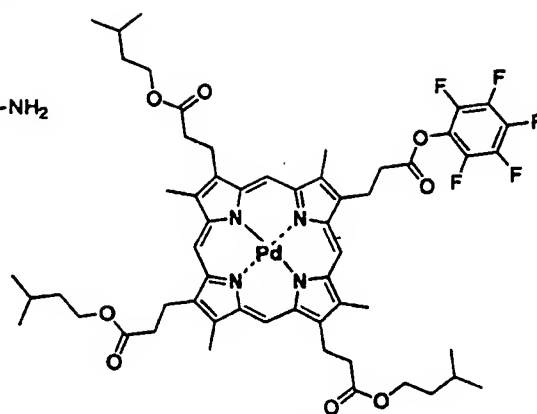
Scheme 3



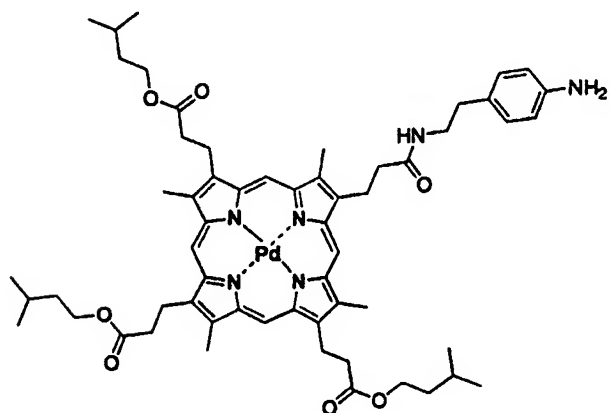
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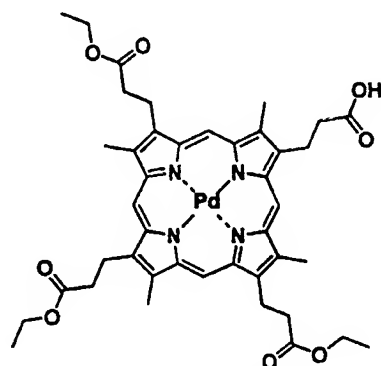
Compound 13



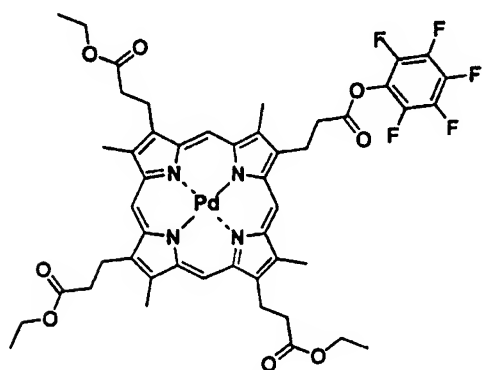
Compound 14



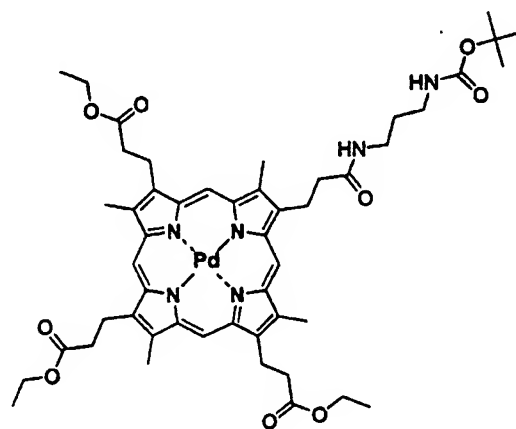
Compound 15



Compound 16



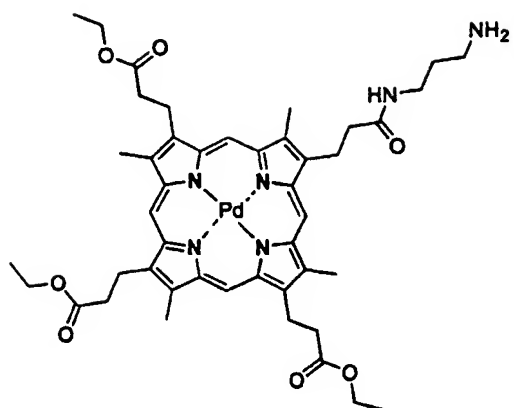
Compound 17



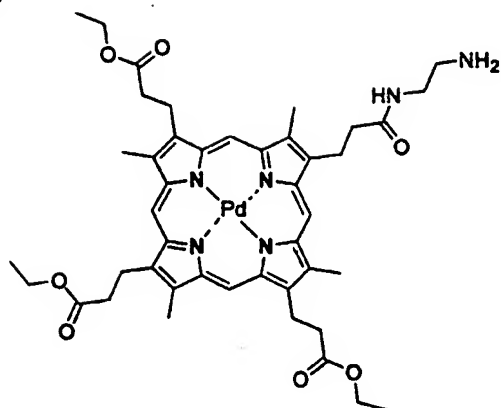
Compound 18

Scheme 4

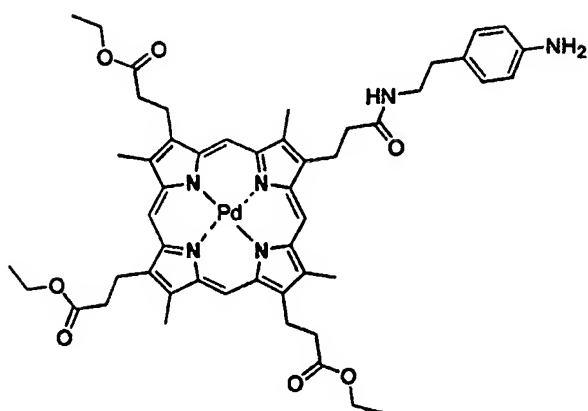
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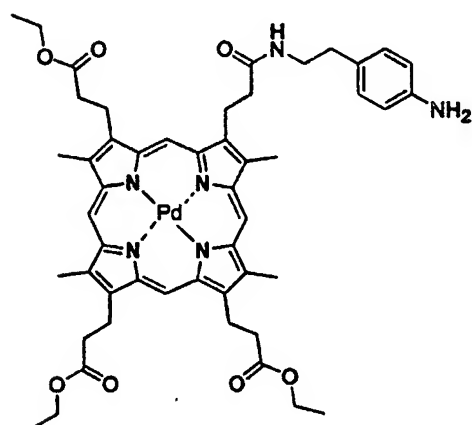
Compound 19



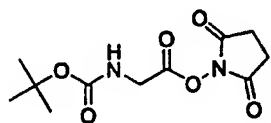
Compound 20



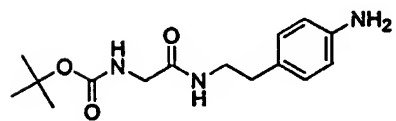
Compound 21



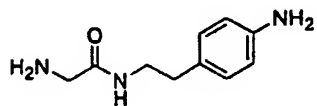
Compound 22



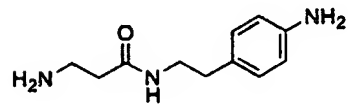
Compound 23a



Compound 23b

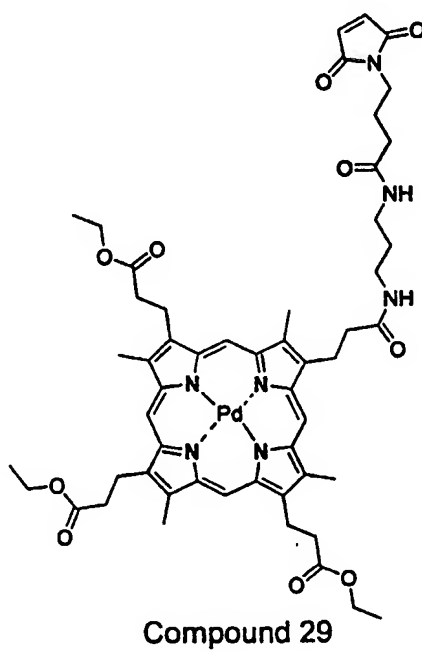
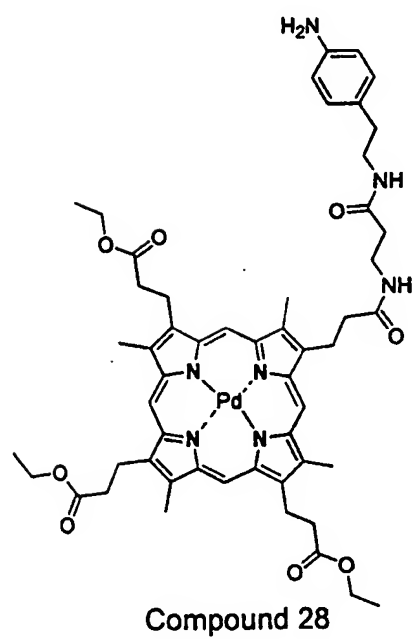
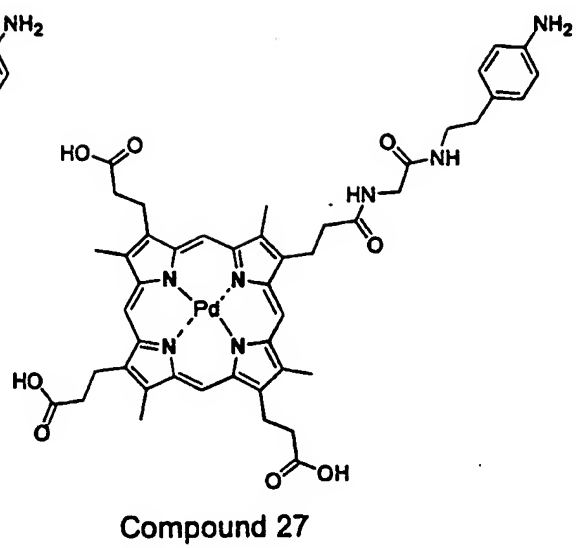
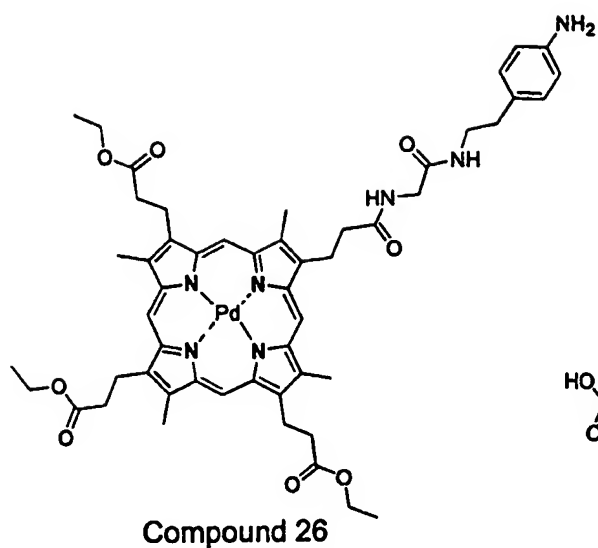


Compound 24

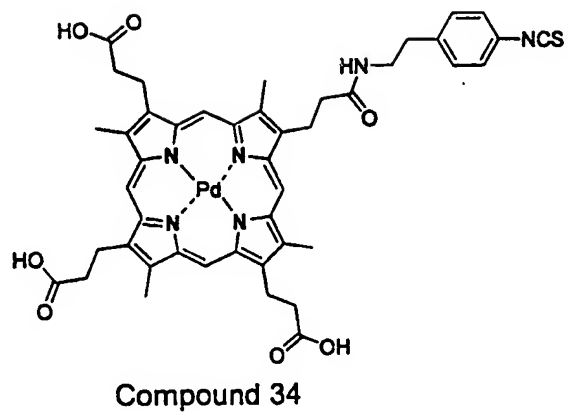
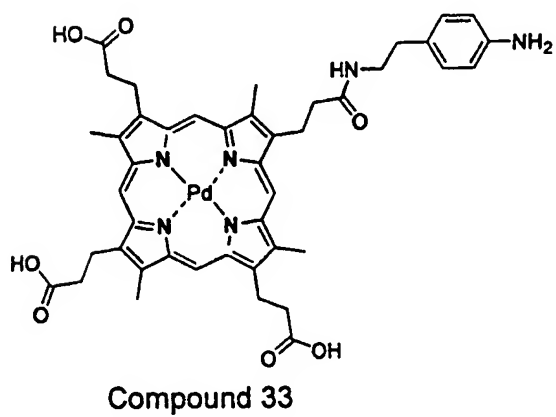
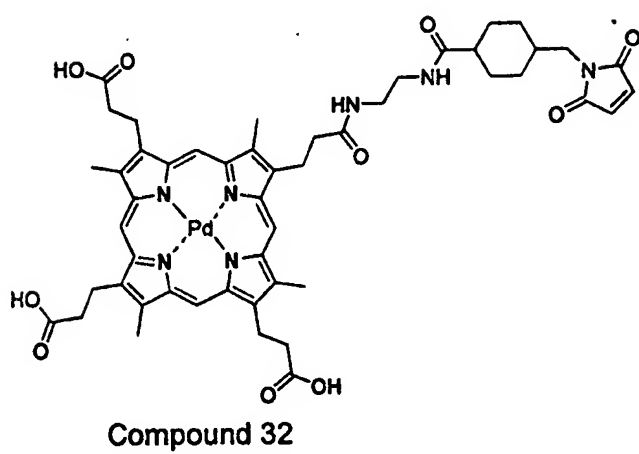
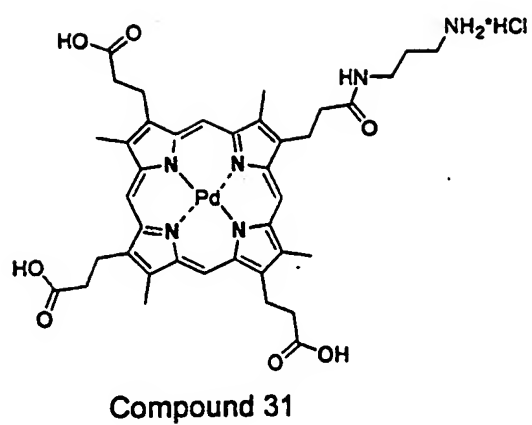
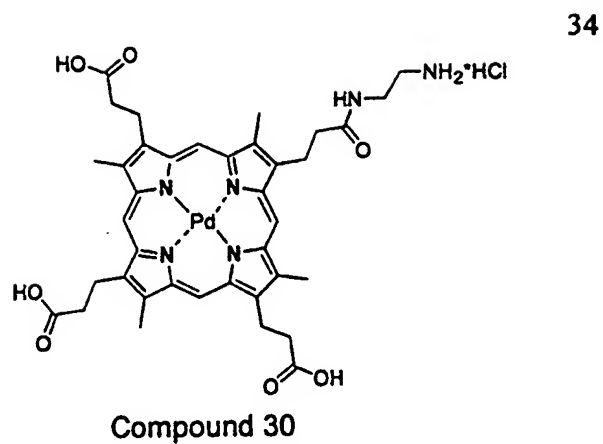


Compound 25

33

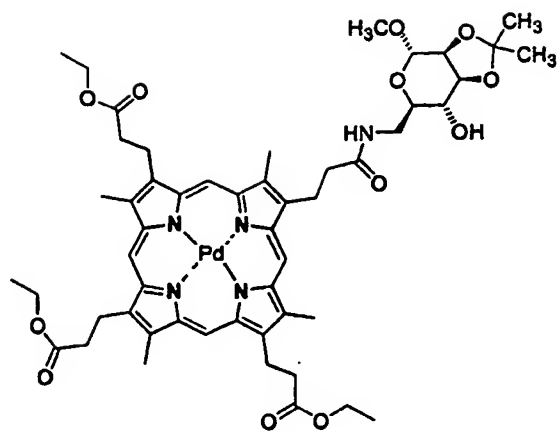


### Scheme 6

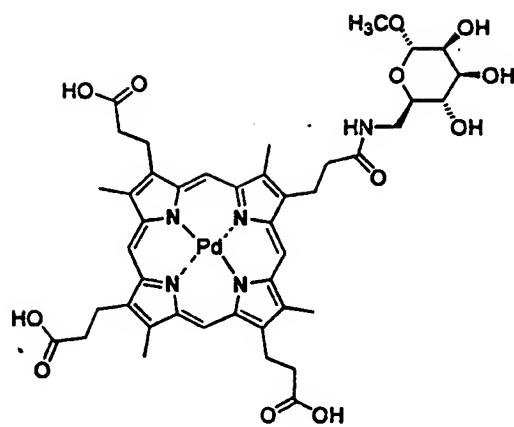


Scheme 7

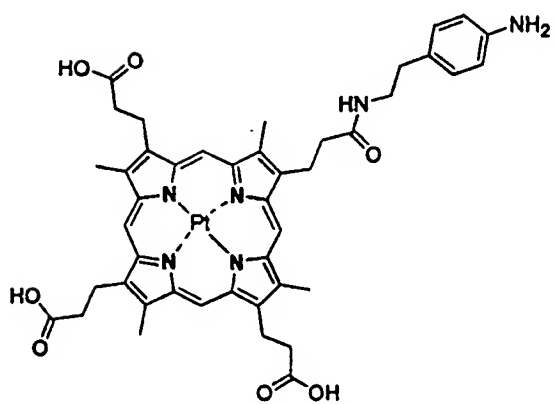
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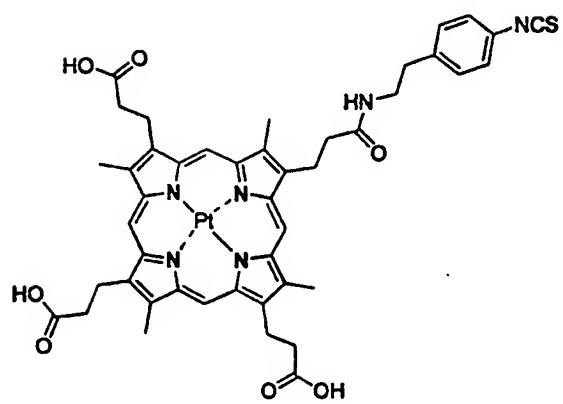
Compound 35



Compound 36



Compound 37

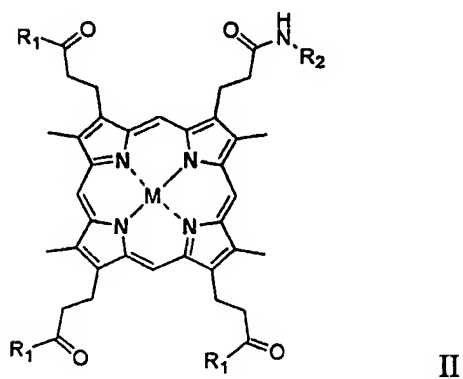
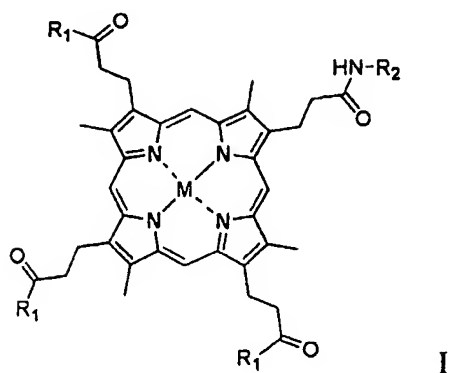


Compound 38

Scheme 8

**Claims:**

1. A compound of formula (I) or (II):



10

characterised in that

M is palladium (II) or platinum (II);

R<sub>1</sub> is -OH, -O<sup>-</sup>X<sup>+</sup> or -OR;

where R is alkyl, aryl, alkaryl or aralkyl group and X<sup>+</sup> is cation

15

R<sub>2</sub> is a chemical moiety being or bearing a functional group, wherein said functional group can be used for selective covalent linkage to other molecules and/or for further chemical modifications.

2. The compound according to Claim 1 characterised in that  $R_2$  is  $-Y-NH_2$  where Y is a chemical moiety.

5 3. The compound according to Claim 1 characterised in that  $R_2$  is  $-Y-Ph-NH_2$  where Y is a chemical moiety and Ph is a phenyl.

4. The compound according to Claim 1 characterised in that  $R_2$  is  $-Y-Z$ , where Y is a chemical moiety and Z is a maleimido group.

10

5. The compound according to Claim 1 characterised in that  $R_2$  is  $-Y-Ph-NCS$  where Y is a chemical moiety and Ph is a phenyl.

6. The compound according to Claim 1 characterised in that  $R_1$  is  $-OH$  or  $-O^-X^+$   
15 and  $R_2$  is  $-(CH_2)_n-Ph-NH_2$  where Ph is a phenyl and n is an integer from 1 to 10.

7. The compound according to Claim 1 characterised in that  $R_1$  is  $-OH$  or  $-O^-X^+$  and  $R_2$  is  $-(CH_2)_n-Ph-NCS$  where Ph is a phenyl and n is an integer from 1 to 10.

20 8. A labelled conjugate comprising a bioaffinity reactant labelled with one or several phosphorescent labels characterised in that the phosphorescent label is a compound according to anyone of the claims 1-7.

9. A conjugate according to Claim 8 characterised in that the bioaffinity reactant is a  
25 biologically active molecule, such as a hapten, biologically active ligand, drug, peptide, oligonucleotide, nucleotide, nucleic acid, polypeptide, protein, antibody, or a fragment of an antibody.

10. A bioaffinity assay method characterised in that at least one of the biospecific  
30 reactants is labelled with the compound according to any of the Claims 1-7.

11. The method according to Claim 10 characterised in that the analyte is  
biologically active molecule, such as a hapten, biologically active ligand, drug,  
peptide, oligonucleotide, nucleotide, nucleic acid, polypeptide, protein, antibody, or  
5 a fragment of an antibody.

12. A cytological or histological staining method characterised in that at least one of  
the biospecific reactants is labelled with the compound according to Claims 1-7.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 99/00898

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07D 473/22, C07F 13/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS Letters, Volume 259, No 2, January 1990, . O.S. Fedorova et al, "Palladium(II)-coproporphyrin I as a photoactivable group in sequence-specific modification of nucleic acids by oligonucleotide derivatives" page 335 - page 337  --	1-12
P,X	The Journal of Histochemistry & Cytochemistry, Volume 47, No 2, February 1999, Richard R. de Haas et al, "Phosphorescent Platinum/Palladium Coproporphyrins for Time-resolved Luminescence Microscopy" page 183 - page 196  --	1,2,8-12

☒ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

- \* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2000	20 -03- 2000
Name and mailing address of the ISA: Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer  Göran Karlsson/Eö Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 99/00898

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Russian Journal of Bioorganic Chemistry, Volume 21, No 4, 1995, O. N. Ponamareva et al, "Synthesis of Phosphorescent Metalloporphyrins with Isothiocyanate Groups" page 296 - page 300  --	1-12
X	Society of Photo-Optical Instrumentation Engineers, Volume 2625, 1989, Andrei F. Mironov, "Synthesis and properties of new chlorin and bacteriochlorin photosensitizers" page 23 - page 32  --	1-12
X	EP 0811626 A1 (BOEHRINGER MANNHEIM GMBH), 10 December 1997 (10.12.97)  --	1-12
A	DE 3827940 A1 (SCHERING AG), 1 March 1990 (01.03.90)  --	1-12
A	EP 0705897 A1 (JOANNEUM RESEARCH FORSCHUNGSGESELLSCHAFT MBH), 10 April 1996 (10.04.96)  -- -----	1-12

# INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/FI 99/00898**

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1, 8-12**  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
**The definition of R2 in claim 1 is not clear and concise, cf. Article 6. Claims 1 and 8-12 have therefore not been fully searched.**

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/FI 99/00898

Patent document cited in search report			Publication date	Patent family member(s)	Publication date
EP	0811626	A1	10/12/97	JP 10067791 A	10/03/98
DE	3827940	A1	01/03/90	AT 128138 T	15/10/95
				AU 622470 B	09/04/92
				AU 3957089 A	15/02/90
				DE 58909444 D	00/00/00
				DK 395289 A	14/02/90
				EP 0355041 A,B	21/02/90
				SE 0355041 T3	
				ES 2077577 T	01/12/95
				GR 3017586 T	31/12/95
				IE 70229 B	13/11/96
				IL 91292 D	00/00/00
				JP 2160789 A	20/06/90
				NO 893245 A	14/02/90
				NZ 230288 A	25/06/92
				PT 91422 A,B	08/03/90
				US 5275801 A	04/01/94
EP	0705897	A1	10/04/96	AT 179746 T	15/05/99
				DE 69418329 D	00/00/00
				US 5718842 A	17/02/98